Harnessing the immunostimulatory properties of oncolytic reovirus for anticancer immunotherapy

Groeneveldt, P.C.

PART B

The effect of preexisting immunity on reovirus therapy

CHAPTER 4

Preexisting Immunity: Barrier or Bridge to Effective Oncolytic Virus Therapy?

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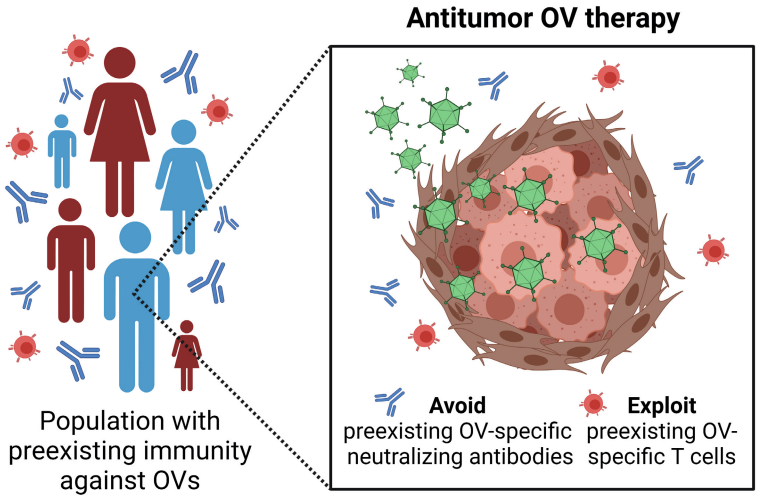
ABSTRACT

Oncolytic viruses (OVs) represent a highly promising treatment strategy for a wide range of cancers, by mediating both the direct killing of tumor cells as well as mobilization of antitumor immune responses. As many OVs circulate in the human population, preexisting OV-specific immune responses are prevalent. Indeed, neutralizing antibodies (NAbs) are abundantly present in the human population for commonly used OVs, such as Adenovirus type 5 (Ad5), Herpes Simplex Virus-1 (HSV-1), Vaccinia virus, Measles virus, and Reovirus. This review discusses (pre)clinical evidence regarding the effect of preexisting immunity against OVs on two distinct aspects of OV therapy; OV infection and spread, as well as the immune response induced upon OV therapy. Combined, this review provides evidence that consideration of preexisting immunity is crucial in realizing the full potential of the highly promising therapeutic implementation of OVs. Future investigation of current gaps in knowledge highlighted in this review should yield a more complete understanding of this topic, ultimately allowing for better and more personalized OV therapies.

List of Abbreviations

Ad5; Adenovirus serotype 5
ADE; antibody-dependent enhancement
ATPP; Antibody-Targeted Pathogen-derived Peptides
BiTE; bispecific T-cell engager
CMV; Cytomegalovirus
CV-A21; Coxsackievirus A21
DAMP; damage-associated molecular pattern
DC; dendritic cells
EBV; Eppstein-Barr virus
EEVs; extracellular enveloped viruses
FcγRs; Fc-gamma receptors
FDA; Food and Drug Administration
HBsAg; Hepatitis B surface antigen
HSV-1; Herpes simplex virus type 1
ISG; interferon-stimulated gene
MHC; major histocompatibility complex
NAbs; neutralizing antibodies
NDV; Newcastle disease virus
NOD; non-obese diabetic
NOG; NOD.Cg-Prkdc^{scid}Il2rg^{tm1Sug}/ShiJic
NSG; NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ
OV; oncolytic virus
OVA; ovalbumin
PAMP; pathogen-associated molecular pattern
TME; tumor microenvironment
T-VEC; talimogene laherparepvec
VSV; Vesicular Stomatitis Virus

GRAPHICAL ABSTRACT



INTRODUCTION

Oncolytic viruses (OVs) are increasingly being recognized as a promising therapeutic modality for the treatment of a variety of cancers (1,2). Selective replication of OVs in cancerous cells, which can either be a result of natural viral tropism or artificially achieved by genetic modification, makes them highly specific antitumor agents with minimal off-target effects. An overview of the most prominently investigated OVs is provided in **Figure 1**. Increasing interest in the clinical potential of OVs has been driven by the Food and Drug Administration (FDA) approval of the modified Herpes Simplex Virus type 1 (HSV-1) talimogene laherparepvec (T-VEC), which was shown to significantly improve survival in patients with late-stage melanoma (3-5). Currently, there is an immense pipeline of over 200 registered clinical trials investigating the therapeutic application of various OVs as single agents or as part of combination therapies (6).

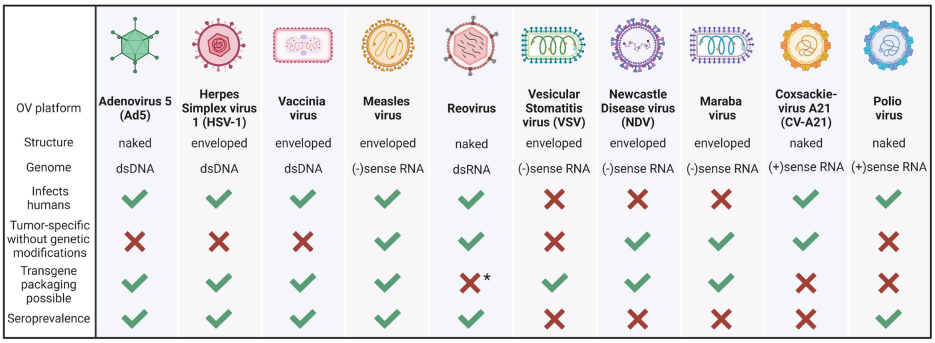


Figure 1. Properties of commonly investigated oncolytic virus (OV) platforms. dsDNA indicates double-stranded DNA. Green checkmarks indicate that a characteristic does apply to the specific OV platform, red crosses indicate that it does not. The presence of seropositivity is derived from clinical trial data (serum samples measured before treatment), or population studies. References for general information about each OV and seropositivity data: Ad5 (7-10), HSV-1 (3,11-13), Vaccinia virus (14-17), Measles virus (18-22), Reovirus (23-29), VSV (30-32), NDV (33), Maraba virus (34), CV-A21 (35-37), Polio virus (38-40). *For Reovirus, only packaging of very small transgenes is possible, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) (41).

Multiple mechanisms of action are known to contribute to the therapeutic efficacy of OVs, as was previously reviewed by us and others (42,43). Direct oncolysis is the result of viral repurposing of the infected cell for the production of viral genomic material and proteins, which eventually results in the release of progeny viral particles through cell lysis (44). Besides direct killing, there is accumulating evidence that shows that OVs can also stimulate strong immune-mediated antitumor effects (45). Local inflammation recruits immune cells to the tumor microenvironment (TME), where viral infection and killing of tumor cells result in the release of both pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) and type I interferons (46). These PAMPs and DAMPs mediate the potent activation of dendritic cells (DCs) for antigen presentation. In combination with high tumor antigen availability due to oncolysis, this constitutes

an OV-induced 'perfect storm' which establishes conditions uniquely favorable for efficient priming and subsequent influx of both virus- and tumor-specific $CD4^+$ and $CD8^+$ T cells (**Figure 2**). Recent investigations into the OV-mediated delivery of immune-stimulating transgenes into the TME, such as cytokines, costimulatory T-cell ligands, checkpoint inhibitors, or even tumor antigens, further illustrate the crucial importance of immunity in the context of OV therapy (47,48). Furthermore, OV therapy can promote the availability of tumor antigens. Most notably, OV-induced oncolysis of infected cells can result in the release of otherwise inaccessible tumor antigens, improving the immune response against cancer cells expressing these epitopes (49). Furthermore, OVs can be employed as so-called oncolytic vaccines, which encode or are coated with tumor antigens to steer the immune response toward antitumor specificity (50,51).

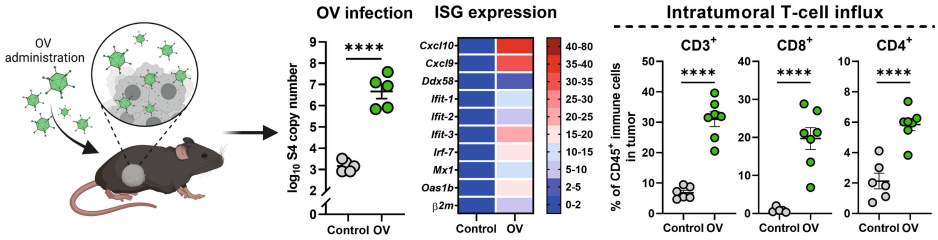


Figure 2. Mechanism of action of immune-stimulatory effects of OVs in the tumor microenvironment. OV administration leads to infection of tumor cells, which induces the upregulation of interferon-stimulated genes (ISGs) including T-cell attracting chemokines. The OV-induced expression of ISGs is followed by an increased influx of T cells into the tumor. Data is derived from studies where oncolytic reovirus is injected intratumorally in immunocompetent C57BL/6J mice bearing murine pancreatic KPC3 tumors (52,53). OV infection and ISG expression was determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and intratumoral T-cell influx was measured by flow cytometry.

Despite the immense therapeutic potential of OVs, some patients do not respond to OV therapy. One of the proposed limiting factors for effective OV therapy is the presence of preexisting immunity in patients (54). Therapeutic application necessitates the use of non-pathogenic OVs, but the fact that they are benign is often a result of the efficient immune response that is induced upon infection. Thus, previous exposure is likely to result in the presence of a potent preexisting immune response. In antiviral immune responses, circulating viral particles are recognized and subsequently neutralized by antibodies, whereas virus-infected cells are targeted by virus-specific cytotoxic $CD8^+$ T cells. Therefore, possible effects of preexisting immunity on OV infection and spread predominantly involve a preexisting humoral response. Indeed, assessment of OV-specific neutralizing antibodies (NAbs) in serum in both the general population and OV clinical trial cohorts, also termed seroprevalence, shows that preexisting immune responses are abundantly present. This is primarily the case for viruses that, besides their application as OVs, also circulate in the human population or are used as vectors for vaccination, such as Adenovirus serotype 5 (Ad5), HSV-1, or Vaccinia virus (**Figure 1**). Seroprevalence is much less common for OVs that mainly infect non-human hosts, such

as Vesicular Stomatitis Virus (VSV) or Newcastle Disease virus (NDV). So far, the general consensus has been that the presence of preexisting immunity decreases OV efficacy by enhancing viral clearance, thus limiting the window of therapeutic action. This has resulted in patient exclusion criteria based on the presence of neutralizing antibodies in some clinical trials, for example (NCT01227551) (55). However, emerging evidence suggests that OV-specific preexisting immunity might actually potentiate antitumor effects in some cases. Thus, a nuanced assessment of the effects of preexisting immunity in the context of OV therapy is warranted.

Here, we provide an overview of the currently available mechanistic insights regarding the effect of preexisting immunity in two distinct phases of OV therapy: 1) OV infection and spread upon administration, and 2) development of the OV therapy-induced immune response, while discussing the many variables that contribute to the effect of preexisting immunity in these phases. Furthermore, we discuss how preexisting immunity can be evaded or even utilized to enhance the therapeutic efficacy of OVs. By shining a light on the complex nature of preexisting immunity in the context of OV therapies, the collection of (pre)clinical data discussed here should prove instructive for future decisions regarding both fundamental investigation as well as the therapeutic application of OVs.

THE EFFECT OF PREEXISTING IMMUNITY ON OV INFECTION AND SPREAD

Until recently, interest regarding the effects of preexisting immunity has been largely focused on the early phases of OV therapy, which comprise the initial infection of tumor cells by the OV, its subsequent spread throughout the circulation, and the dissemination to distant tumors and tissues. Although neutralizing antibodies for commonly used OVs are present in the human population as well as cancer patients (**Figure 1**), their effect on the therapeutic efficacy of OVs is highly dependent on many variables, including the route of administration and the specific OV platform used.

Intratumoral OV therapy is largely unaffected by preexisting immunity

In the field of OV therapy, local versus systemic delivery is a huge topic of debate (56). Local, intratumoral delivery of OVs is in clinical practice for T-VEC (57,58) and is often used in preclinical studies to ensure efficient delivery to the tumor site (59). Theoretically, intratumorally administered OVs might be less accessible to preexisting antibodies than circulating OVs (**Figure 3A**), although this could vary depending on tumor vascularization (60). Direct cell-to-cell spread after infection with several OVs, including HSV-1, Vaccinia virus, and Measles virus, was shown to be unaffected by the presence of neutralizing antibodies in both *in vitro* and *in vivo* contexts (61,62). However, several other studies have reported that preexisting immunity can limit intratumoral viral replication or spread (63-65). For example, induction of preexisting immunity by intramuscular exposure to

Ad5 before intratumoral injection of this OV into subcutaneous HPD-1NR pancreatic carcinomas, resulted in rapid clearance of viral load from the tumor in hamsters (66).

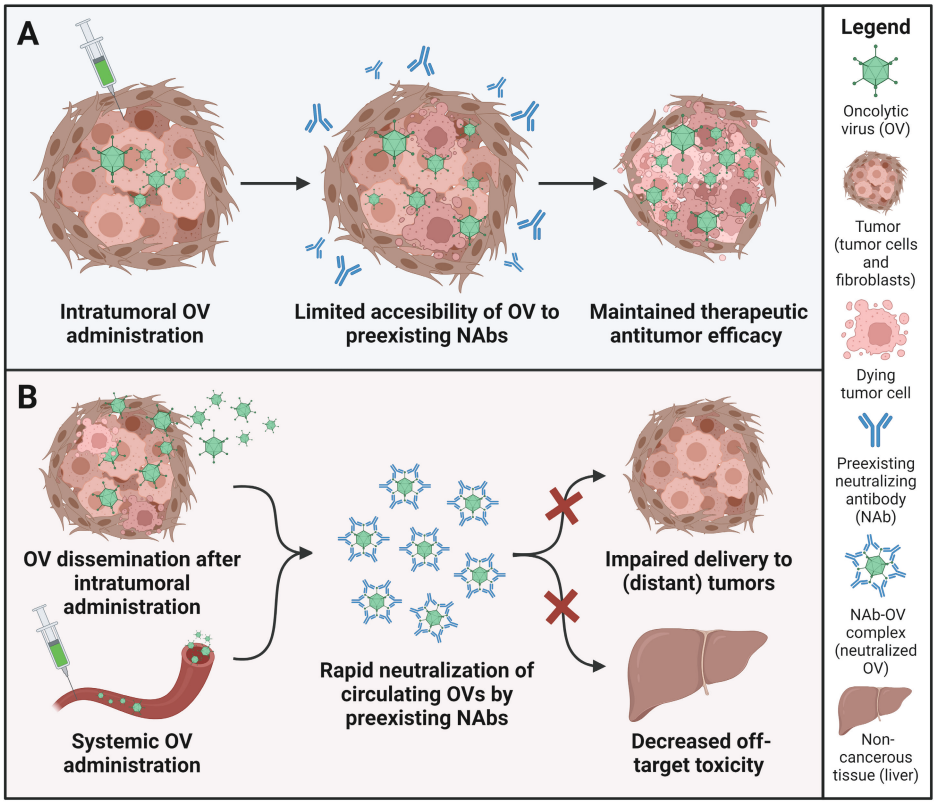


Figure 3. Route of administration contributes to the effect of preexisting neutralizing antibodies (NAbs) on OV efficacy. (**A**) Intratumorally injected OVs might be less accessible to preexisting NAbs (in blue), leading to maintained therapeutic antitumor efficacy. (**B**) OVs that disseminate into the circulation after intratumoral injection, as well as OVs that are systemically administered, are susceptible to rapid neutralization. This can limit the delivery efficiency to (distant) tumors, but also decrease off-target toxicity.

Interestingly, although preexisting immunity against Ad5 and other OVs including HSV-1 and Coxsackievirus A21 (CV-A21) can result in reduced intratumoral viral replication, preexisting immunity against these OVs does not mitigate the OV-induced effect on primary tumor growth or animal survival upon intratumoral OV therapy (63-69). These results highlight the discrepancy between viral replication and therapeutic efficacy in the setting of intratumoral administration (70). Indeed, clinical trials investigating the efficacy of various OVs with high seroprevalence that were injected directly into a variety of readily accessible tumors, such as melanomas, have been relatively successful (4,55,71-73). One of these trials, investigating the efficacy of intratumoral Ad5 treatment of pancreatic ductal adenocarcinoma, showed there was no significant correlation between preexisting anti-Ad5 antibody titers and changes in tumor size upon therapy (74). Thus, both the preclinical and clinical data suggest that, even though OV replication

might be decreased, preexisting immunity should generally not be considered an obstacle to primary tumor clearance in the setting of intratumoral OV therapy.

Rapid neutralization of OVs shed by infected tumors prevents viral dissemination

Although intratumoral OV therapy consists of direct injection of OVs into the tumor microenvironment, spillover and viral shedding as a consequence of oncolysis will introduce OVs into the circulation. These circulating OVs are readily accessible to preexisting antibodies and thus subject to neutralization, which can impact OV therapy in a variety of ways (**Figure 3B**). For instance, in the case of multiple tumors, preexisting immunity might prevent intratumorally administered OVs from disseminating to distant tumors. An example of this was shown to occur upon injection of CV-A21 into one of two bilateral subcutaneous YUMM 2.1 melanomas in immunocompetent C57BL/6 mice (69). For naive animals, viral genomic material was present in the blood and both tumors, but intraperitoneal preexposure to CV-A21 completely precluded viral recovery from the circulation and the distant tumor. Likewise, another study showed that passive immunization with Vaccinia-specific immunoglobulins strongly reduced dissemination to lung, bone, and lymph node metastases in BALB/c mice upon injection of a primary 4T1 mammary carcinoma with luciferase-expressing Vaccinia virus (75). As such, preexisting immunity is likely detrimental to therapeutic efficacy in a setting of metastatic disease, where therapy should affect both the injected and distant tumors.

Importantly, preexisting NAbs can reduce toxicity associated with intratumoral OV therapy by limiting viral dissemination to off-target tissues. This was investigated in a study using an intratumoral injection of subcutaneous PymT-induced breast adenocarcinoma with a luciferase-expressing replication-deficient Ad5 in FVB/n mice (63). Here, intranasal exposure to Ad5 before OV therapy strongly reduced luciferase activity in the liver, which is a major site of Adenovirus off-target toxicity, while only slightly reducing transgene expression in the tumor. Similar results were obtained for intratumoral treatment of subcutaneous HaK kidney tumors with an Ad5 OV in Syrian hamsters (64). Here, intramuscular preexposure to Ad5 completely abrogated recovery of viral genome copies from the liver and lungs, as well as infectious virus from the liver, whereas naive animals exhibited dissemination to these tissues and active viral replication in the liver. Importantly, tumor growth was similarly inhibited for both naive and preexposed animals. Thus, it appears that NAbs prevent OV dissemination to distant tumors upon intratumoral therapy, but can also be beneficial by limiting dissemination and infection of off-target tissues.

The efficacy of systemic OV therapy is abrogated by preexisting immunity

Clinically speaking, systemic OV administration is often preferable to intratumoral injection, as it limits patient discomfort and allows for the treatment of tumors that are not easily accessible (76). However, since therapeutic efficacy in this context is completely dependent on dissemination via the circulation, preexisting immunity

represents a major hurdle to this route of administration (**Figure 3B**). Indeed, the preclinical efficacy of most systemically-administered OV therapies, including Measles virus, VSV, HSV-1, and Ad5, is severely abrogated by preexisting immunity (77-80). For example, a study investigating the efficacy of intravenous VSV-GFP treatment in BALB/c mice bearing subcutaneous CT26 colon carcinomas demonstrated that intravenous VSV exposure before OV therapy completely abrogated transgene expression and recovery of infectious virus from the tumor, which was not observed in naive animals (81). Similar attenuation was observed upon passive immunization with antibody-containing serum, but not for animals receiving a transfer of T cells from donor mice exposed to VSV. Passive immunization with purified Ad5-specific antibodies was also shown to inhibit intratumoral Ad5 replication and clearance of subcutaneous LNCaP prostate cancer tumors in BALB/c nude mice treated intravenously with Ad5, while Ad5 treatment demonstrated antitumor activity in a setting without Ad5 NAbs (80). As such, the accessibility of these systemically administered OVs to NAbs appears to be the main reason for their diminished therapeutic efficacy in an immunized host.

The specific site of intravenous delivery might be an important consideration for therapeutic outcome, as it influences the effect of preexisting immunity on OV efficacy. This was shown for HSV-1 therapy in BALB/c mice carrying hepatic metastases established by subcapsular injection of CT26 colon carcinoma cells (82). Here, intraperitoneal preexposure attenuated HSV-1-induced tumor clearance upon tail vein, but not portal vein delivery of HSV-1. As delivery into the portal vein reduces the distance to its target, it likely minimizes the window in which preexisting antibodies can abrogate therapeutic efficacy through the neutralization of OVs. Thus, this observation supports a model in which the required distance of OV dissemination is inversely related to the attenuating effect of preexisting immunity. Together, these studies support the role of preexisting antibodies as a likely contributing factor to the limited efficacy of clinical trials investigating systemic OV delivery and show that nuanced consideration of delivery sites is warranted.

EVADING PREEXISTING IMMUNITY FOR IMPROVED OV INFECTION AND SPREAD

To improve the infection and spread by OVs, many studies have explored modifications of OV therapy to evade neutralization by NAbs (54). Especially in the context of systemic therapy, such strategies might strongly increase therapeutic efficacy.

Cell carriage can rescue the efficacy of systemic OV therapy despite preexisting immunity

Avoiding recognition of OVs by neutralizing antibodies might be achieved by utilizing infected cells as 'Trojan horses' to deliver OVs to tumors (**Figure 4A, C**). Early clinical trials demonstrated that systemically delivered Reovirus was able to reach and actively

infect distant tumors, despite the presence of Reovirus-specific NAbs (83). Interestingly, replication-competent Reovirus could be recovered from circulating PBMCs, granulocytes, and platelets but not plasma. This suggests that immune cell carriage can be employed for shuttling and handing off OVs to distant tumors, as a means to evade OV clearance by neutralizing antibodies. Indeed, mechanistic studies have shown that Reovirus can be internalized by various immune cells, including DCs and T cells (84,85). One study assessed the consequences of cell carriage by subcutaneously implanting B16 melanomas, treating C57BL/6 mice intravenously with either free or cell-carried Reovirus, and then assessing the number of metastatic colonies in the tumor-draining lymph node (86). For both naive and Reovirus preexposed animals, Reovirus-loaded mature DCs and T cells outperformed free OVs in limiting lymph node metastases, likely as a result of more efficient draining and thus viral delivery to lymph nodes by immune cells. Similarly, T cells loaded with Measles virus facilitated delivery of Measles virus to tumors in the presence of NAbs (87). Other studies have investigated stem cells as potential OV carriers, as they are naturally resistant to chemotherapeutic drugs and can survive in the tumor microenvironment (88). As an example of such a strategy, Ad5-infected neural stem cells were less susceptible to *in vitro* serum neutralization and led to more efficient *in vivo* infection of intracranial GL261 gliomas when delivered in multiple cycles, compared to naked OVs (89).

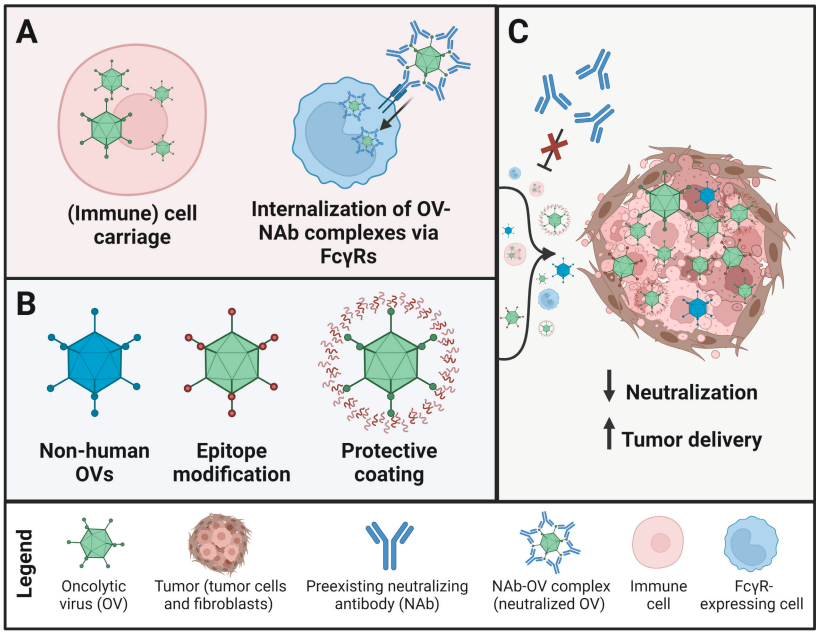


Figure 4. Strategies to evade neutralization by preexisting neutralizing antibodies (NAbs). (**A**) OVs can be carried by various (immune) cells, such as dendritic cells (DCs), T cells or stem cells to avoid neutralization. Alternatively, OV-NAb complexes can be internalized by cells expressing antibody-binding Fc-gamma receptors. (**B**) Usage of non-human OVs, epitope modification or a protective coating to decrease recognition and clearance by NAbs. (**C**) Employment of evasion strategies described in (A) and (B) lead to decreased neutralization and improved delivery of the OV to the tumor.

Interestingly, when using a cell carrier system for OV delivery, the presence of NAbs might even be beneficial. For instance, antibody-Reovirus complexes can effectively be internalized by human monocytes and delivered to tumor cells, resulting in infection and lysis of Mel-624 melanoma cells. This internalization is mediated via the antibody-binding Fc-gamma receptors (FcγRs), expressed on the surface of monocytes and other immune cells (90). Similarly, antibody-neutralized CV-A21 was shown to be ineffective at killing Mel-624 cells *in vitro* unless carried and handed off by monocytes (90). Furthermore, A549 lung carcinoma cell lines artificially expressing FcγRs have been shown to internalize antibody-neutralized Ad5 (91). The antibody-dependent enhancement (ADE) of viral infection through internalization of antibody-virus complexes by FcγR-expressing cells has been described to occur for a range of viruses, such as Influenza virus, Measles virus, Coronaviruses, and most notably Flaviviruses (92,93). In contrast to Reovirus, these viruses can efficiently replicate in their carrier cells, ultimately resulting in cell death. Since this broadens viral tropism and eliminates immune cells, ADE is often associated with poorer disease outcomes. As such, the ability of some OVs to productively replicate in FcγR-expressing cells might preclude them from beneficial cell carriage, as it would result in the rapid elimination of carrier cells before they can facilitate viral dissemination to distant tumors. Nevertheless, it appears that delivery of OVs via (immune) cell carriage could be a promising new approach for systemic delivery of OVs, especially in preexposed individuals.

Non-human OVs demonstrate oncolytic activity towards human tumors but are less susceptible to neutralization

Another way to avoid recognition by preexisting immune responses is the use of alternative viral strains, which are sufficiently different from their human-infecting homologs but also display oncolytic effects (**Figure 4B, C**). The capacity of non-human OVs to kill human tumor cells has been demonstrated for various viruses, such as an HSV-1 virus derived from goats that was able to replicate in different human cell lines and induce apoptosis (94,95). Additionally, Adenoviruses isolated from non-human primates were shown to effectively infect and kill a wide range of human cancer cell lines *in vitro*, while not being neutralized by pooled human donor serum (96). Similarly, an avian Reovirus was able to infect hepatocellular carcinoma cells and induce apoptosis *in vitro* but is likely less susceptible to neutralization in humans, since structural analysis demonstrated that its neutralizing epitopes were distinctly different from its human homolog (97). Other examples of non-human virus species that are in development as oncolytic agents have been described elsewhere (98,99). The (pre)clinical efficacy of most of these non-human viruses remains to be proven, but they represent an attractive alternative to currently used OVs.

Genetic modification limits neutralization by OV-specific preexisting antibodies

Alternatively, antibody-binding sites of OVs can be altered by genetic modification, preventing neutralization by preexisting antibodies (**Figure 4B, C**). For example, the introduction of point mutations in the gD glycoprotein of HSV-1 was shown to result

in increased resistance to *in vitro* neutralization by monoclonal antibodies (100). More radical modification is also possible by exchanging surface glycoproteins of OVs with those from other viruses with lower rates of preexposure in the population. This so-called envelope exchange has been utilized for the generation of chimeric Measles virus strains with surface proteins originating from the Canine Distemper virus, which retain their oncolytic activity *in vitro* and *in vivo* (77,79,101). Indeed, this modified Measles virus demonstrated potent oncolytic antitumor efficacy in athymic nude mice bearing intraperitoneal SKOV3.ip1 ovarian cancers and passively immunized with measles-immune human antibody serum, while the efficacy of the non-modified Measles virus was strongly diminished (79). Similar chimerism has also been explored for Ad5, by switching its serotype to that of the related Ad3 or Ad35 to evade neutralization (102-104).

Shielding or coating of OVs prevents immune recognition

Modification of neutralizing epitopes on OVs or the use of OVs from other hosts thus appear promising for the evasion of preexisting immunity present in the population. Nevertheless, both modified and non-human OVs will likely still be affected by the antiviral immune response induced by repeated therapeutic administrations. Thus, shielding surface epitopes of OVs with a non-immunogenic coat to prevent recognition might be an alternative strategy (**Figure 4B, C**). This can be achieved by genetic modification of the OV, as was shown for the insertion of an albumin-binding domain in the main capsid protein of Ad5 (105). Intravenous administration of a luciferase-expressing Ad5 virus into nude mice bearing subcutaneous B16-CAR melanomas that were intraperitoneally preexposed to Ad5 led to complete neutralization, as the Ad5-mediated luciferase expression within tumors was completely abolished. In contrast, the albumin-binding Ad5 did not suffer from significant loss of luciferase signal in tumors. Similarly, in nude mice bearing subcutaneous A549 or Sk-mel28 tumors that were intraperitoneally preexposed to Ad5, the oncolytic antitumor efficacy of intravenously administered albumin-binding Ad5 was maintained while the Ad5 without the albumin-binding domain was completely inefficacious. As another example, Vaccinia virus has been successfully modified to increase the release of so-called extracellular enveloped viruses (EEVs) upon infection, which have an additional membrane layer and are thereby less susceptible to immune-mediated clearance compared to Vaccinia virus particles themselves (75). This EEV-enhanced Vaccinia virus displayed improved spread to metastases in the lungs and lymph nodes after intratumoral delivery in BALB/c mice inoculated with 4T1 tumors in the mammary fat pad, compared to a Vaccinia virus variant that was less capable to produce EEVs. Similarly, a significant survival advantage was provided by the EEV-enhanced strain over the wild-type virus in BALB/c mice bearing subcutaneous JC tumors.

Alternatively, OVs can be artificially coated by the attachment of ionic polymers, graphene sheets, or liposomes to shield them from antibody recognition (78,106). For example, multilayer ionic polymer coating of Measles virus resulted in improved

control of subcutaneous LL/2-CD64 lung cancer tumors compared to the non-coated virus in Measles-preimmunized C57BL/6N mice (107). In another study, shielding of Ad11 using a hybrid membrane comprised of artificial lipid membranes and red blood cell membranes protected the virus from neutralizing antibodies, prolonged its circulation, and enhanced its antitumor efficacy in the murine TC1 lung cancer model (108). Further (pre)clinical evaluation of the strategies described above would be interesting to optimally enhance the efficacy of (systemically delivered) OV therapy in preexposed patients.

EFFECTS OF PREEXISTING IMMUNITY ON THE OV THERAPY-INDUCED IMMUNE RESPONSE

Besides viral replication and oncolysis, the induction of a potent immune response is a second, but equally important pillar of OV therapy (45) (see also section 1). However, if and how the presence of preexisting immunity also affects the OV-induced immune response remains underexplored. Here, we gathered (pre)clinical evidence that describes the effect of preexisting immunity regarding the induction of virus- and tumor-specific immune responses.

Repeated OV exposure can limit the induction of a tumor-specific immune response

Indications that preexisting immunity can affect OV-induced immune responses can be derived from studies utilizing multiple dosages of OVs. Specifically, it has been shown that homologous boosting regimens impair the induction of a tumor-specific T-cell response, in contrast to heterologous prime-boost schedules utilizing a combination of distinct OV platforms. An example of this was shown for intratumoral OV therapy of hamsters with subcutaneously implanted HaK kidney tumors or HPD-1NR pancreatic carcinomas (109). In both models, a heterologous treatment schedule comprising three intratumoral Ad5 injections followed by three intratumoral Vaccinia injections displayed significantly superior antitumor efficacy compared to 6 doses of either virus alone. This heterologous OV therapy resulted in improved induction of tumor-specific T cells compared to treatment with either virus alone, and these T cells were responsible for therapeutic efficacy since the depletion of $CD3^+$ T cells completely abrogated the antitumor effect of this combination therapy. OVs encoding a transgene appear to be similarly affected by dosage regimens. For instance, in a CT26 metastasis model where tumors express β-galactosidase, two intravenous doses of either β-galactosidase-expressing Vaccinia virus or the related β-galactosidase-expressing Fowlpox virus resulted in inferior overall survival compared to sequential treatment with both viruses (110). Heterologous boosting led to higher β-galactosidase-specific $CD8^+$ T-cell responses compared to homologous boosting, and homologous boosting was associated with the induction of a strong antiviral antibody response.

Although repeated OV administration can hamper the OV-induced tumor-specific T-cell response, evidence for the mechanisms underlying this problem remains elusive. This phenomenon could simply be explained by lower clearance of the OV by NAbs, but another possible explanation could be derived from the immunodominance of previously encountered viral T-cell epitopes. Besides the notion that most viral epitopes are inherently more immunogenic than most tumor epitopes, an OV-specific T-cell response is boosted upon the reintroduction of previously recognized viral epitopes. Both aspects might result in an immunodominant OV-specific T-cell response over the tumor-specific T-cell response (**Figure 5**). This phenomenon, sometimes referred to as 'original antigenic sin' (111), has been extensively studied for vector-based vaccines and Influenza infections but has not gained a lot of attention in the field of OV research (112). Nevertheless, as similar viral strains are often used in both fields, data showing problematic viral epitope immunodominance for vector vaccines highlights current gaps in OV research and might indicate shared mechanisms. Of note, it could be that different OV platforms vary in their inherent immunogenicity, making them more or less dominant over the tumor-specific immune response. Indeed, research on viral vectors has shown that viral backbones can differ in the type and potency of immune responses they induce (113), indicating the same might be true for OVs.

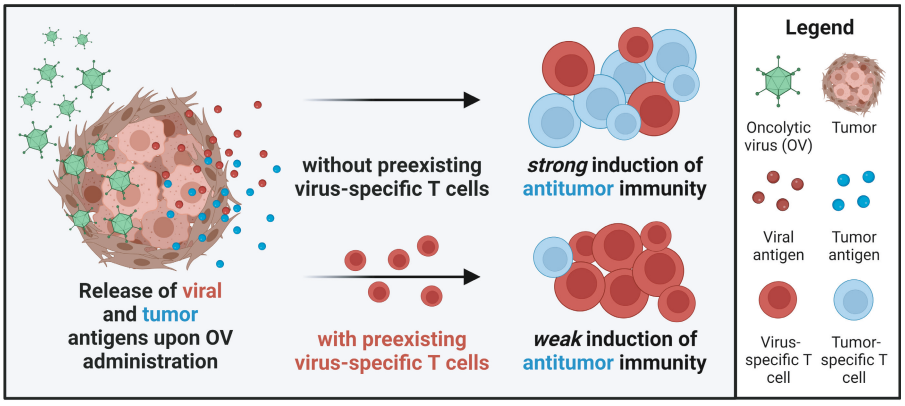


Figure 5. Immunodominance of OV-specific T-cell responses over tumor-specific T-cell responses. Viral epitopes are often more immunogenic compared to most tumor epitopes. Additionally, in the setting of preexisting immunity or repeated dosage, the preexisting OV-specific T-cell response is boosted upon repeated recognition of the viral epitopes. These combined aspects may result in an impaired induction of an antitumor T-cell response compared to a strong virus-specific T-cell response.

The possible immunodominance of the viral backbone over transgenes could especially be relevant for OVs which encode tumor antigens. For instance, investigation of intramuscular delivery of Hepatitis B surface antigen (HBsAg) and ovalbumin (OVA) antigen in BALB/c mice using an Ad5-based vector revealed prior exposure to Ad5 strongly reduced the HBsAg- and OVA-specific $CD8^+$ T-cell responses (114). Instead, isolated $CD8^+$ T cells were mainly reactive to Ad5 epitopes. Skewing of immunity towards an antiviral response was replicated in antibody-deficient $IgH^{-/-}$ mice, indicating it is the

established Ad5-specific cellular immunity, and not the Ad5-specific humoral response, that limits the priming and expansion of HBsAg/OVA-specific $CD8^+$ T cells. Similarly, induction of $CD4^+$ and $CD8^+$ T-cell responses specific for an Influenza virus antigen, which was intramuscularly delivered using a Vaccinia virus vector, was completely abrogated by prior exposure to Vaccinia virus (115). Highlighting the relevance of such preclinical observations, clinical data suggests similar immunodominance occurs in humans. For example, several trials of Ad-vectored vaccines have reported correlations between preexisting Ad-specific $CD4^+$ T cells (116,117) or antibodies (118) and strongly decreased induction of $CD4^+$ T-cell, $CD8^+$ T-cell and antibody responses directed against the delivered vaccine antigen. Although these studies utilized Adenovirus to deliver Ebolavirus and HIV epitopes irrelevant to OV therapy, these observations might be relevant to the field of OV research. Furthermore, the discussed data on OV boosting regimens might suggest that preexisting immunity could also affect responses to tumor antigens released after oncolysis, due to the simultaneous release of viral epitopes.

Intramuscular, intravenous, and intratumoral OV administration are likely to result in distinct dynamics of viral epitope exposure to the immune system and thus influence the development of antiviral immunodominance, but a direct comparison of routes of administration has yet to be performed. Regardless of the underlying mechanisms, heterologous prime-boost regimens appear to be beneficial for some OV therapies by improving tumor-specific immune responses and tumor clearance. Consequently, clinical trials of such strategies are promising and currently ongoing. For example, sequential systemic therapy with Ad5 and Maraba virus, both encoding the tumor antigen MAGE-A3, showed preclinical efficacy and is currently being tested for the treatment of advanced metastatic solid tumors and non-small cell lung cancer (NCT02285816, NCT02879760) (119-121).

Preexisting OV-specific immunity can also improve therapeutic anticancer efficacy by enhancing tumor-specific T-cell responses

The data described above suggested that OV administration might result in the dominance of OV-specific T-cell responses over tumor-specific T-cell responses upon repeated exposure. However, other studies suggest that preexisting OV-specific immunity does not hamper, but can actually promote the induction of a systemic tumor-specific immune response. An example of this was shown for immunocompetent C57BL/6J mice with subcutaneously implanted bilateral B16.F10 melanomas, of which one was injected with NDV (122). In this setting, prior subcutaneous footpad exposure to NDV led to improved control of tumor size as well as extended survival upon intratumoral NDV treatment, even though viral replication was compromised. For both the injected and distant tumor, the ratio of conventional $CD4^+$ T cells over regulatory T cells as well as the expression of genes related to immune-mediated cytotoxicity were strongly increased by preexposure to NDV. In the distant, but not the injected tumor of preexposed animals, an increase in $CD8^+$ T-cell influx could be observed, which was not the case for the distant tumors of naive animals. Prior NDV exposure did not significantly increase

the amount of virus-specific $CD8^+$ T cells in the spleen but instead caused a strong increase in the amount of tumor-specific $CD8^+$ T cells. $CD8^+$ T-cell depletion completely abrogated the antitumor effect of NDV in immunized mice, suggesting that $CD8^+$ T cells were indispensable for the therapeutic efficacy of NDV in a preexposed setting. Similar effects of preexisting immunity on therapeutic OV efficacy were recently shown for the intratumoral treatment of BALB/c mice with subcutaneously implanted bilateral CT26 colon carcinomas using a highly modified HSV-1, expressing several cytokines and a PD-L1 blocking peptide (123). Control of both the injected and distant tumors was improved by subcutaneous preexposure to HSV-1, as was overall survival. Strikingly, the outgrowth of the distant tumor was completely unaffected by intratumoral OV therapy of the local tumor in naive animals, showing preexisting immunity was required for systemic efficacy in this setting. Gene expression profiling of tumors again revealed a skewing toward cytotoxic and inflammatory responses. Additionally, isolated splenocytes from preexposed mice were more reactive to tumor cells compared to splenocytes from naive animals, indicating an increased induction of tumor-specific immunity.

Thus, it appears that preexisting immunity can also promote the induction of a tumor-specific immune response upon therapy with these OVs. These tumor-specific responses have a systemic impact with efficacy on distant tumors and could thus have the potential to treat metastatic disease. Whether this phenomenon extends to other OV platforms and its underlying mechanisms, however, remains to be explored. One possibility could be that preexisting antiviral $CD4^+$ T cells aid the development of tumor-specific $CD8^+$ T cell responses. $CD4^+$ T-cell help has been well established as a crucial factor in the induction of robust $CD8^+$ T-cell responses but is generally considered to be restricted to responses specific to the same antigen (124). Nevertheless, some studies have indicated that $CD4^+$ T cells might also mediate more general immune-stimulating effects upon activation by their cognate antigen, such as an increase in naive lymphocyte recruitment to lymph nodes (125). Indeed, it was recently shown in C57BL/6 mice that were intramuscularly vaccinated with tetanus toxoid before intratumoral OVA-coated Ad5 therapy, that additional coating of the Ad5 with major histocompatibility complex (MHC) class II-restricted tetanus toxoid peptides led to increased infiltration of tumor-specific $CD8^+$ T cells into subcutaneous B16.OVA melanomas (126). As the tetanus toxoid coating resulted in potent stimulation of preexisting pathogen-specific $CD4^+$ T-helper cells, it appears likely that pathogen-specific $CD4^+$ T-cell help can potentiate tumor-specific $CD8^+$ T-cell responses. In another study, it was revealed that prior vaccination against poliovirus substantially improved the antitumor efficacy of intratumoral polio treatment in C57BL/6 mice bearing murine melanoma B16.F10 tumors, and that this antitumor effect was mediated by the recall of $CD4^+$ T cells and the induction of tumor-specific T cells that could delay tumor outgrowth in naive mice after adoptive cell transfer (127). So far, preexisting virus-specific $CD4^+$ T cells have been largely overlooked in the OV research field, but these observations suggest that they might play an important part in modulating the OV-induced immune response, especially in a setting where preexposure has occurred.

EXPLOITING PREEXISTING VIRUS-SPECIFIC IMMUNITY FOR EFFECTIVE ANTICANCER IMMUNOTHERAPY

Regardless of the induction of a tumor-specific immune response in a preexposed setting, increasing amounts of evidence suggest that preexisting antiviral effector responses might also be engaged to directly contribute to tumor clearance and thus therapeutic efficacy. Studies have shown that antiviral $CD8^+$ T cells commonly survey a range of both murine and human tumors, including melanomas, brain metastases, endometrial, lung, and colorectal cancers (128,129). Upon immune cell profiling, tumor-specific $CD8^+$ T cells found in patient tumors expressed high levels of T-cell exhaustion, likely as a result of chronic antigen exposure in the tumor (129). $CD8^+$ T cells specific for common viral pathogens, such as Cytomegalovirus (CMV), Eppstein-Barr virus (EBV), or Influenza virus, on the other hand, exhibited phenotypes more in line with active effector cells. Indeed, virus-specific T cells, as determined by staining with HLA tetramers specific for these viruses, could be potently activated after isolation from tumor tissue by providing relevant viral peptides (128). Various strategies are described to employ antiviral T cells for anticancer therapy, either by reactivation using their cognate antigens, or in a specificity-independent manner.

Preexisting antiviral T cells can be activated and engaged for anticancer therapy

The engagement of preexisting antiviral T cells for antitumor activity is an appealing avenue for immunotherapy, in particular for the treatment of low-immunogenic tumors (**Figure 6**). One way to achieve this is by delivering viral epitopes into the tumor, resulting in the activation of antiviral T cells present in the tumor microenvironment. For example, preexisting Reovirus-specific $CD8^+$ T cells, induced by vaccination with a synthetic viral peptide containing the Reovirus $CD8^+$ T-cell epitope, were efficiently recruited into subcutaneous KPC3 pancreatic tumors upon intratumoral injection of Reovirus (53). In this study, the presence of this preinstalled pool of Reovirus-specific effector cells significantly delayed tumor outgrowth after intratumoral Reovirus administration, an effect not observed when Reovirus was administered to naive animals. Similar effects on tumor growth were observed in animals that were immunized with Reovirus before vaccination, showing that vaccine-mediated boosting of preexisting Reovirus-specific $CD8^+$ T cells can improve OV therapeutic efficacy. Similarly, intratumoral delivery of the Vaccinia virus-derived B8R protein by a recombinant adeno-associated virus reactivated preinduced Vaccinia-specific $CD4^+$ and $CD8^+$ T cells and retarded outgrowth of murine DT6606 pancreatic tumors (130).

Besides direct infection with a virus, other innovative strategies can also be employed to reactivate virus-specific T cells. Although these studies often investigate the use of non-OV-specific T cells, these observations should also be instructive for the employment of preexisting OV-specific T cells. For example, injection of B16 melanomas with the viral peptide SIINFEKL resulted in improved tumor control and survival over an irrelevant peptide in C57BL/6J mice that had previously received a transfer of OT-1 $CD8^+$ T cells

which target this epitope (128). Similarly, intratumoral injection of murine CMV (MCMV)-derived T-cell epitopes triggered the expansion of MCMV-specific $CD4^+$ and $CD8^+$ T cells in TC1-bearing immunocompetent mice that were preexposed to MCMV (131). Injection of MHC-I-restricted MCMV epitopes into TC1 tumors induced a T-cell/IFN-γ signature, delayed tumor outgrowth, and improved survival. Expanding on such an approach is the idea that the conjugation of virus-derived epitopes to tumor-targeting antibodies might improve their specificity and facilitate systemic efficacy. This was demonstrated in a study where CMV-derived epitopes conjugated to an antibody targeting the tumor antigen MMP14 could be used for efficient recruitment of preexisting antiviral $CD8^+$ T cells towards various MMP14-expressing tumors (132). This resulted in improved control of orthotopic MDA-MB-231 breast tumors, as well as orthotopic SNU-475 liver or subcutaneous MGH-1 lung tumors.

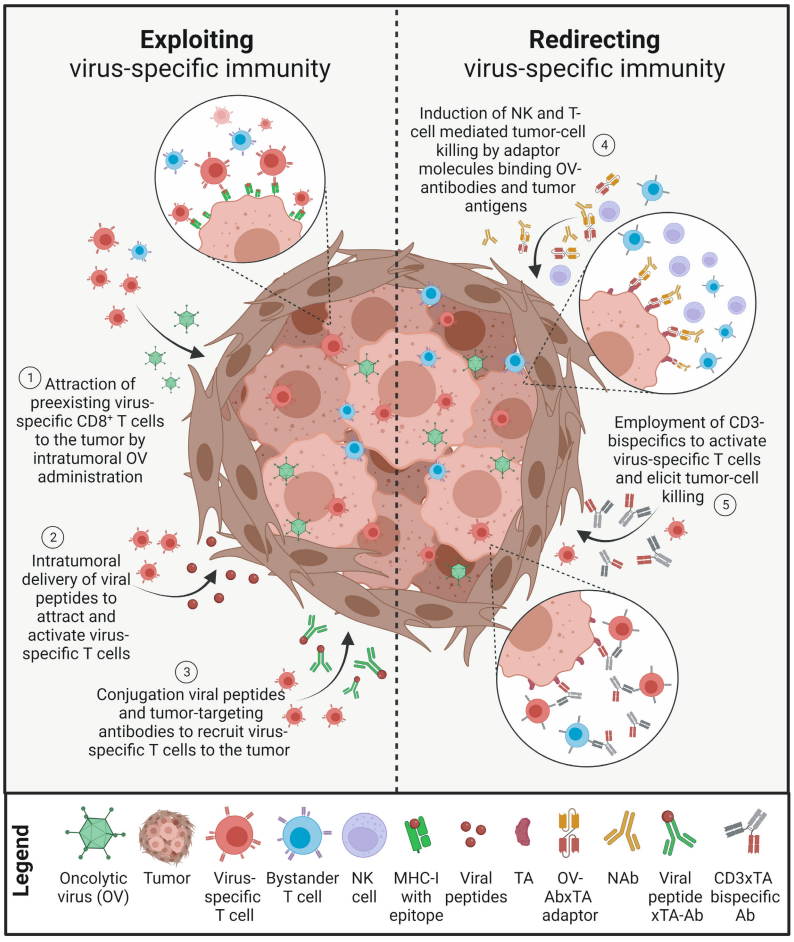


Figure 6. Strategies to exploit or redirect (preexisting) virus-specific T cells for antitumor immunotherapy. Multiple avenues can be employed to exploit the specificity of (oncolytic) virus-specific immunity for anticancer immunotherapy. (1) Preexisting OV-specific T cells can be attracted to the tumor by intratumoral OV administration and activated by presentation of OV >>

>> epitopes on the surface of tumor cells in MHC-I proteins. (2) Intratumoral delivery of viral peptides leads to activation of intratumoral virus-specific T cells. (3) Complexes of viral peptides together with a tumor-targeting antibody can recruit OV-specific T cells to the tumor. (4) Adaptor molecules binding to both OV-specific antibodies and tumor antigens induce NK- and T-cell-mediated killing of tumor cells. (5) Utilization of CD3-bispecific antibodies transforms OV-specific T cells into tumor-attacking T cells. NK; natural killer, MHC-I; major histocompatibility molecule class I; TA; tumor antigen, Ab; antibody.

A similar principle was applied in a model where immunodeficient female non-obese diabetic (NOD).Cg-Prkdc^{scid}Il2rg^{tm1Sug}/ShiJic (NOG) mice bearing MDA-MB-231 breast cancer xenografts received an adoptive transfer of expanded human EBV-specific CD8$^+$ T cells, which were subsequently directed to the tumor by use of immunoconjugates called Antibody-Targeted Pathogen-derived Peptides (ATPPs) (133). Here, MHC class I peptides are conjugated to antibodies specific for a tumor antigen that is expressed on the tumor cell surface. This tumor-specific delivery of EBV peptides activated EBV-specific T cells and delayed tumor outgrowth in combination with PD-1 checkpoint blockade. Similarly, in NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice bearing the same MDA-MB-231 tumors, CMV-specific T cells could be redirected to exert antitumor efficacy via a CD8$^+$ T-cell epitope-delivering antibody (termed TEDbody), which was engineered to deliver a viral MHC-I epitope peptide into the cytosol of target tumor cells by fusion with a tumor-specific cytosol-penetrating antibody (134).

Thus, delivery of viral epitopes into the tumor microenvironment, through a variety of ways, can be utilized to engage preexisting antiviral T cell populations for antitumor effect. An exciting strategy involves the exploitation of 'molecular mimicry', where preexisting virus-specific T cells can demonstrate cross-reactivity toward tumors after restimulation with tumor-specific antigens that display high similarities to their cognate viral antigens (135). For instance, in a cohort of melanoma patients with high anti-CMV antibody levels, it was suggested that molecular mimicry between CMV and tumor antigens played a role in the response to anti-PD1 therapy blockade by activation of cross-reactive T cells. Another enticing opportunity is the reactivation of T cells that are induced by exposure to a common virus or established antiviral vaccines, which have already been abundantly tested for clinical safety and are administered to a majority of the human population. As examples of this, T cells specific for Influenza virus (136), Yellow Fever virus (137), or even SARS-CoV-2 (138) might be employed for anticancer therapy.

Redirecting the specificity of preexisting virus-specific responses for their use as anticancer effector cells

As an alternative approach to using the specificity of preexisting antiviral immune responses, the inherent specificities could also be redirected to the tumor by using bispecific molecules (139). Such retargeting of preexisting virus-specific antibodies and T cells for antitumor activity using bispecific molecules might be used to improve OV efficacy (140) (**Figure 6**). For instance, a recent study described the design of a bispecific adaptor molecule containing an Ad5 antibody-binding epitope and a domain

that binds polysialic acid, a surface adhesion molecule associated with a range of cancers (141). Immunocompetent C57BL/6 mice were immunized with Ad5 to develop anti-Ad5 antibodies, which were subsequently recruited to the tumor with the bispecific adaptor molecule. This treatment led to improved tumor control and survival of mice with subcutaneous polysialic acid-expressing MC38 colon carcinomas, CMT-64 lung carcinomas, and B16F10 melanomas compared to naive mice. Further studies in MC38 tumors established a model in which the retargeted Ad5 antibodies recruited and activated NK cells, which mediated initial tumor cell killing through antibody-dependent cellular cytotoxicity (ADCC) and thereby induced the priming of a tumor-specific $CD8^+$ T-cell response (142).

Besides virus-specific antibodies, preexisting OV-specific T cells can also be directly recruited for antitumor efficacy using CD3-bispecific molecules (also known as bispecific T-cell engagers (BiTEs)). For instance, intratumoral Reovirus administration to subcutaneous KPC3 pancreatic tumors expressing tumor antigen TRP1 led to a strong influx of virus-specific $CD8^+$ T cells, which could be subsequently engaged for delayed tumor growth by intraperitoneal administration of a bispecific antibody targeting both CD3 and TRP1 (52). When tested in a bilateral model, this combination therapy led to delayed tumor growth for both the injected and non-injected distant tumors, showing such strategies could be efficacious in a setting of metastatic disease. Current undertakings in this field especially involve the use of OVs encoding BiTEs, where the OV acts both as an immunostimulatory agent, as well as a vector for BiTE delivery into the tumor (143). Together, these results showcase the potential of bypassing the specificity of preexisting antiviral immunity using bispecific molecules for effective anticancer therapy.

CONCLUDING REMARKS

In this review, we discussed how preexisting immunity against OVs can act as a barrier, but also as a bridge to effective anticancer therapy. As is evident from the data described here, a preexisting OV-specific humoral response against commonly-used OVs might limit viral replication and spread, especially when the OV is administered intravenously. Importantly, even for OVs that do not abundantly circulate in the human population, the observations discussed here are highly relevant, as therapeutic regimens usually entail multiple OV administrations. Each dose will invariably lead to the development of an antiviral immune response that modulates the efficacy of the next round of therapy.

Effects of preexisting immunity on OV infection and spread have been relatively well explored and suggest that, although various OV modifications can help evade a preexisting immune response, a nuanced case-by-case assessment appears warranted and variables such as the location of the tumor(s), the specific OV used, as well as

the route of OV administration should be taken into account. For example, treatment of a single, easily accessible tumor by intratumoral OV injection would likely not be compromised by preexisting immunity. In the case of metastatic disease, on the other hand, the therapeutic efficacy of both intratumoral and systemic OV administration will be strongly limited by preexisting immunity, making modifications to evade it beneficial or even necessary. Currently, a variable that remains largely unexplored in this context is the confounding effect of tumor location. As discussed, the distance between the site of intravenous OV administration and the target tumor appears to modulate the effect of preexisting immunity on therapeutic efficacy (82), indicating administration sites should be optimized based on tumor localization.

While, generally speaking, preexisting humoral immunity is considered to be a barrier to effective anticancer OV therapy and should be circumvented, preexisting OV-specific cellular immune responses might rather be considered a beneficial factor for OV therapy. Additionally, the route of OV administration, which has been abundantly explored and discussed here in the context of OV infection and spread, remains strongly underappreciated regarding its effect on the induced immune response. Vaccine studies have uncovered clear evidence showing that the site of administration is a crucial determinant of the type and quality of subsequently induced responses (144), highlighting the need for evaluation of this factor in OV research. Regardless of its effects on the induction of a tumor-specific immune response, exciting novel data suggests preexisting OV-specific adaptive immunity can be engaged for direct antitumor effects. However, careful investigation is warranted, since preexisting OV-specific T cells might also be involved in inducing viral clearance (66). Further research in the field of OV research should elucidate how OV replication, the OV-induced immune response, and the ultimate therapeutic effects of OVs all interrelate, and how both preexisting humoral and adaptive immunity influence these aspects.

In conclusion, consideration of preexisting immunity is crucial in realizing the full potential of the highly promising therapeutic implementation of OVs. Future investigation of the current gaps in knowledge highlighted here should yield a more complete understanding of the topic, ultimately allowing for better and more personalized OV therapies.

DECLARATIONS

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